B5

(12) UK Patent Application (19) GB (11) 2 183 660 (13) A

(43) Application published 10 Jun 1987

(21) Application No 8627520

(22) Date of filing 20 Nov 1985

(30) Priority data

(31) 801014 (32) 22 Nov 1985 (33) US

(71) Applicant
Ciba-Geigy AG,

(Incorporated in Switzerland),

Klybeckstrasse 141, 4002 Basle, Switzerland

(72) Inventors
Ingo Potrykus,
Raymond Douglas Shillito,
Mary-Dell Chilton

(74) Agent and/or Address for Service
Kenneth D. Sparrow, Ciba-Geigy PLC, Patent Department, Tenax Road, Trafford Park, Manchester M17 1WT

(51) INT CL[4]
C12N 15/00

(52) Domestic classification (Edition I)
C3H 644 657 B7V
C6F 10X GD

(56) Documents cited
GB A 2159173 EP A 0086537
GB A 2140822 WO 84/02920
EP 0164575
EMBO J. Vol 4 pages 1367 1985
EMBO J. Vol 3 page 2717 1984
NATURE Vol 291 page 513 1981
Note: GB A 2159173 and EP 0164575 are equivalent;

(58) Field of search
C3H
C6F
Selected US specifications from IPC sub-class C12N

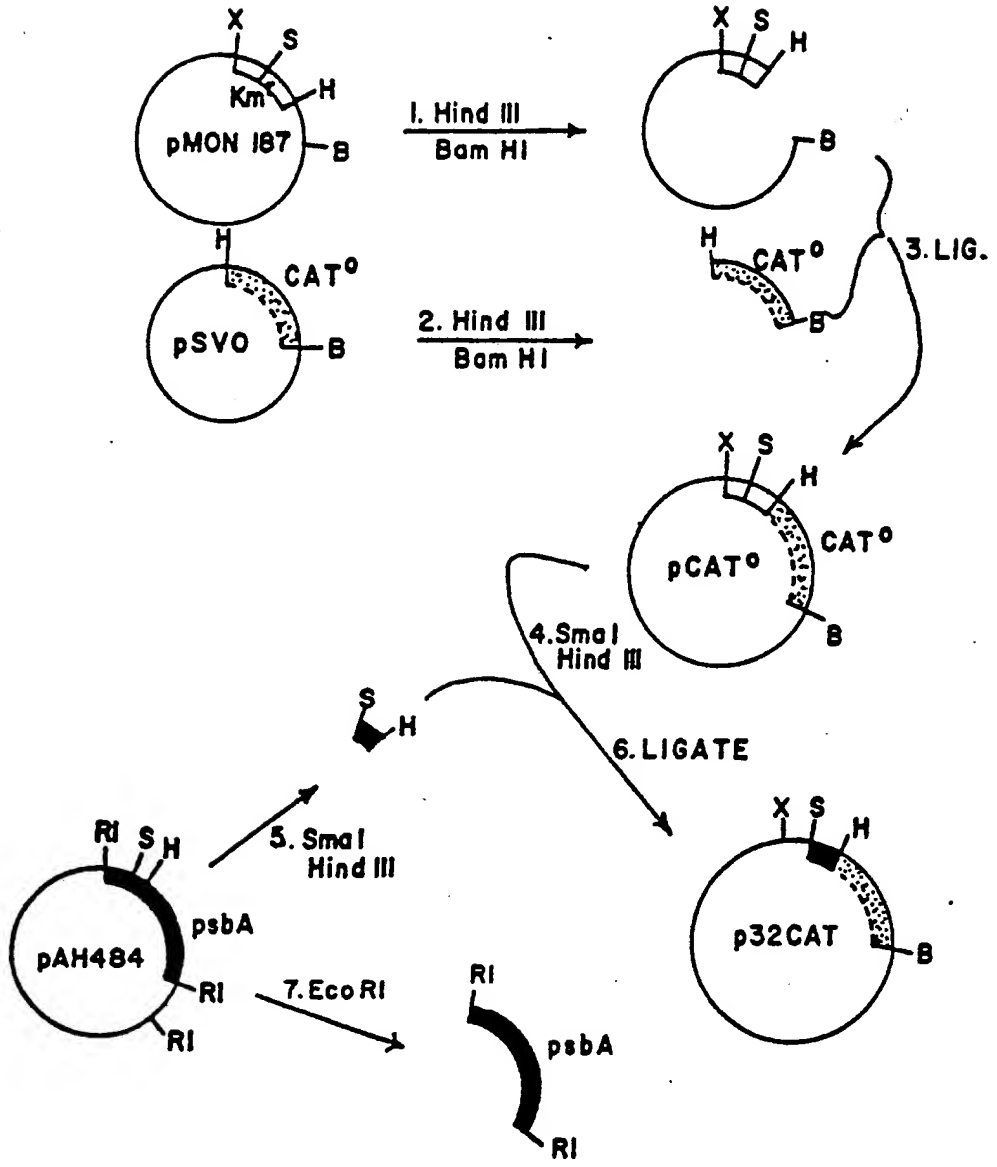
(54) Direct insertion of DNA into plastids and mitochondria

(57) The process for introducing DNA comprising one or more genes having a promoter that is functional in plastids or mitochondria directly into plastids or mitochondria in a plant cell protoplast comprises exposing the DNA to the protoplast in a medium in which the DNA penetrates the protoplast and the plastids or mitochondria in the protoplast for a time sufficient to permit such penetration, without exposing the protoplast to a pathogen.

Preferably the DNA confers herbicide resistance to chloroplasts.

GB 2 183 660 A

FIGURE 1


X
S
Km
H
pMON 187
B
1. Hind III
Bam HI
X S H
B
H
CAT°
pSVO
B
2. Hind III
Bam HI
H CAT°
B
3. LIG.
X S H
pCAT°
CAT°
B
4. SmaI
Hind III
S
H
6. LIGATE
RI S H
5. SmaI
Hind III
pAH484
psbA
RI
RI
7. Eco RI
RI
psbA
RI
X S H
p32CAT
B

2183660

FIGURE 2

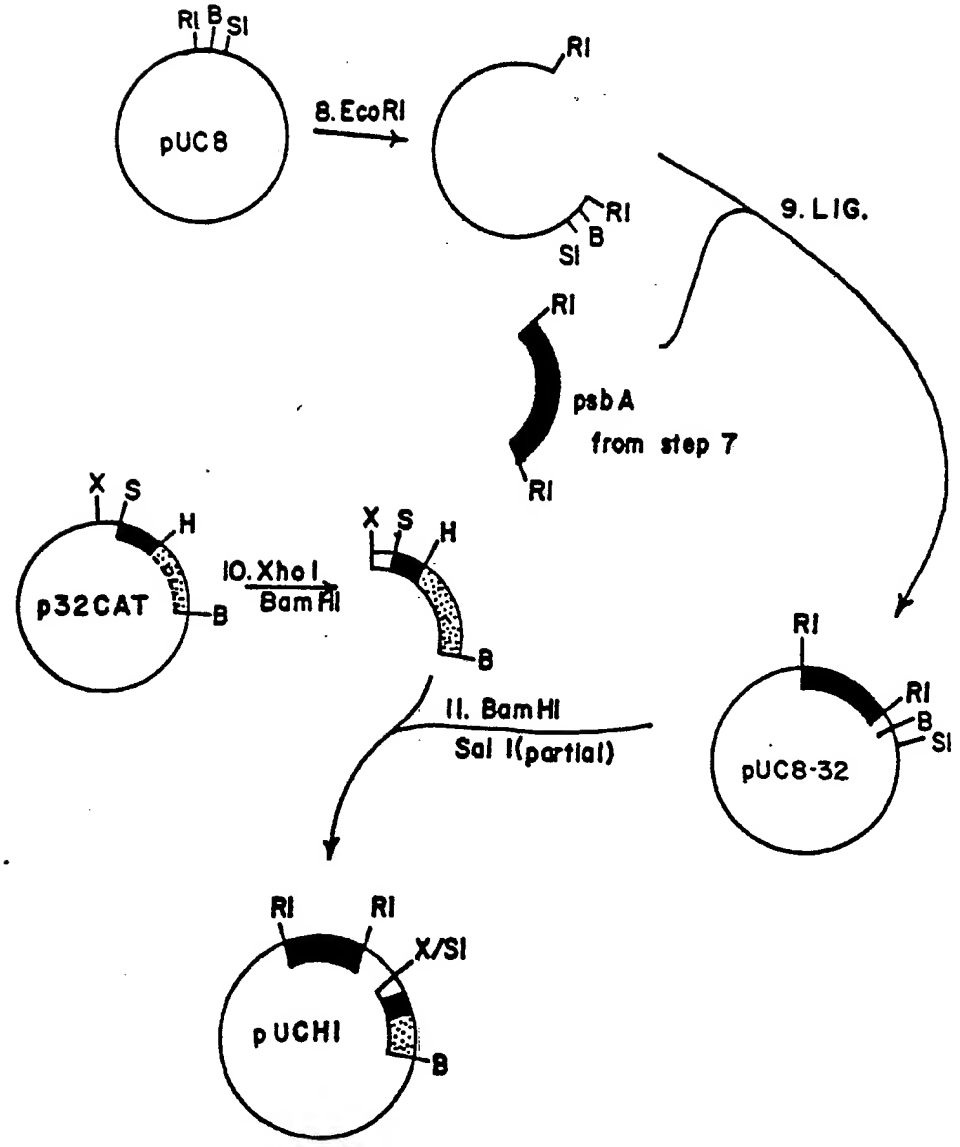
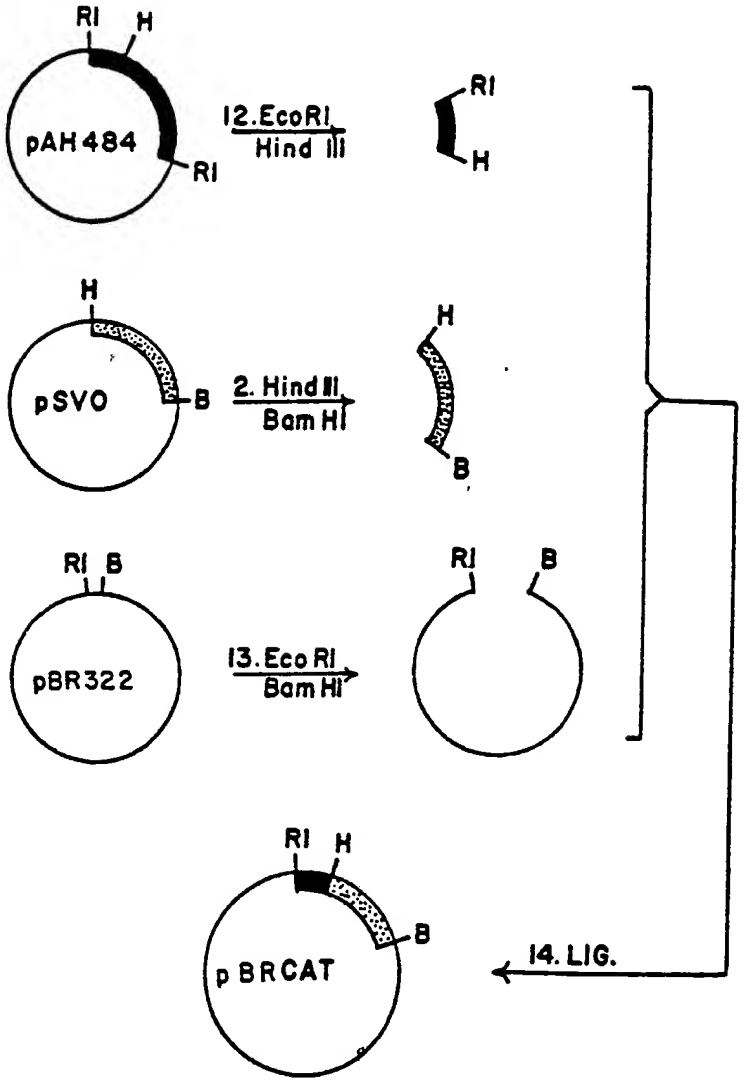


FIGURE 3


RI
H
pAH484
RI
12. EcoRI
Hind III
RI
H
H
pSVO
B
2. Hind III
Bam HI
H
B
RI
B
pBR322
13. Eco RI
Bam HI
RI
B
RI
H
B
p BRCAT
14. LIG.

SPECIFICATION

Direct inserti n of DNA into plastids and mitochondria

The pres nt invention is directed to a method for direct gene transfer to plastids or mitoch ndria, and especially chloroplasts, in plant cell protoplasts. Plants are, for the purposes of the present invention, unicellular or multicellular organisms that undergo photosynthesis. Plant cell protoplasts are plant cells that have been partially or completely divested of cell wall material by treatment with cellulolytic enzymes.

Chloroplasts are the targets for certain classes of herbicides, for example the triazine herbicides. It has recently been discovered that atrazine, a member of the triazine class of herbicides, acts on a 32kd polypeptide that is encoded by a chloroplast gene called psbA (Hirschberg et al., 1984). A single nucleotide substitution in the coding region of psbA leading to a single amino acid substitution in the 32kd polypeptide confers resistance to atrazine. Such mutations occur in weeds, for example *Amaranthus hybridus* and *Solanum nigrum*.

It would be desirable to be able to introduce genes directly into the plastid and mitochondrial genomes of plants and especially crop plants. This would enable one to introduce new and desirable traits into such plants. For example, genes conferring herbicides resistance are needed in herbicide sensitive chloroplasts in order to produce herbicide resistant plants.

Until now, attempts have generally been made to manipulate the nuclear genome of plant cells in order to achieve resistance or tolerance towards herbicides that are active in chloroplasts. Such a strategy, however, confers only tolerance to these herbicides, but not true resistance. It has not been considered possible to confer true resistance toward herbicides active in chloroplasts on crop plants by direct gene transfer, since it has not been thought possible to transform plastids such as chloroplasts by this method.

An additional disadvantage of introducing a gene conferring a desirable trait such as herbicide resistance into the nuclear genome of plants arises from the ability of some plants to pollinate weeds. Such crosses provide a mechanism for the desirable trait to be introduced into weeds.

The most common method used for introducing genes into the nuclear chromosomes of plant cells is the infection of the cells with pathogens, such as *Agribacterium* containing Ti plasmid vector systems. (Barton et al., 1983; Chilton et al., 1985). Such methods suffer from disadvantages due to the infection. Disadvantages include limited host specificity and the necessity of freeing transformed plant cells from the pathogen used for the transformation procedure. Concerns have been raised about the release of such pathogens in the environment (Roberts, 1985).

De Block et al. (1985) have reported the use of an Agrobacterium Ti plasmid vector system for introduction of a gene encoding antibiotic resistance into the chloroplast genome of tobacco protoplasts from which whole cells and a complete fertile plant could be regenerated. However these investigators found that in the absence of antibiotics, the gene was unstable and was lost over a short period of time. Maintenance of plants under antibiotic selection has no practical utility.

Methods of transforming plant cell protoplasts directly with naked linear or circular plasmid DNA are also known (Paszkowski et al., 1984); Schilperoort et al., 1983). Such methods do not require infecting plant cells with a pathogen. Until now, however, such methods have been limited to nuclear transformation.

There is a need for methods that will permit the direct transfer of useful genes into the genome of plastids and mitochondria in order to be able to confer advantageous new properties on plant cells without infecting the cells with a pathogen while preventing such properties from being transferred to weeds. There is a further need for a method of direct transfer of genes into plastids and mitochondria of plant cells and whole plants stably so that expression of the gene is not lost during the development of the crop in the field.

Thus, the principal object of the present invention is to provide a method for directly introducing and maintaining genes in the genome of plastids and mitochondria, especially of chloroplasts without infecting the cells with a pathogen. It is a further object of the present invention to produce whole plants containing genetically engineered plastids or mitochondria under conditions in which the genetically engineered trait or traits are stably maintained and expressed.

This and other objects as will become apparent from the following description have been achieved by providing a process for introducing DNA comprising one or more genes having a promoter that is functional in plastids or mitochondria directly into plastids or mitochdondria in a plant cells protoplast, the process comprising exposing the DNA to the protoplast in a medium in which the DNA penetrates the protoplast and the plastids or mitochondria in the protoplast for a time sufficient to permit such penetration, without exposing the protoplast to a pathogen.

Figures

Figur 1 is flow diagram summarizing the steps for constructing pCAT° and p32CAT.

Figur 2 is a flow diagram summarizing the st ps for constructing pUCH1.

Figure 3 is a flow diagram summarizing the steps for constructing pBRCAT.

Legends for Figures

X =XhoI
S =SmaI
H =HindIII
B =BamHI
RI =EcoRI
SI =SalI
LIG.=ligation of T4 DNA ligase

X/SI indicates a site where ligation of an XhoI site to a SalI site has produced a hybrid site cleavable by neither enzyme.

CAT represents the coding region for chloramphenicol acetyl transferase. CAT° indicates the promoterless form of the CAT gene.

In Figures 1–3, the psbA gene is indicated by a black bar, and its promoter by a black box.

In Figures 1–3, the CAT coding region is stippled.

Definitions

The following definitions apply to the specification and claims:

A "gene" is a DNA sequence comprising a promoter and a transcribed sequence. The promoter, which in most cases is not transcribed, causes the gene sequence following it, and in some cases flanking it, to be transcribed into RNA may or may not be translated into a polypeptide. If the RNA is translated, the RNA is called mRNA. The DNA sequence of the gene corresponding to the mRNA as well as the mRNA itself, comprises a 5' untranslated region, a coding region, and a 3' untranslated region. Only the coding region is translated into a polypeptide.

The "active portion" of a DNA sequence constitutes that portion responsible for the function of the DNA sequence. Some examples of active portions of DNA sequences include the RNA polymerase binding site and transcription initiation signal (TATA box) of a promoter, the ribosome binding site and translation initiation signal of the 5' untranslated region, the coding sequence and the transcription stop signal and polyadenylation signal of the 3' untranslated region. The various spacer regions, in which the DNA sequence is unimportant, are not considered to be active DNA sequences.

A "variant" of a natural DNA sequence is a modified form of the natural sequence that performs the same function. The variant may be a mutation, or may be a synthetic DNA sequence, and is substantially homologous to the corresponding natural sequence. A DNA sequence is substantially homologous to a second DNA sequence if at least 70 %, preferably at least 80 %, and most preferably at least 90 % of the active portions of DNA sequence are homologous. Two different nucleotides are nevertheless considered to be homologous in a DNA sequence of a coding region for the purpose of determining substantial homology if the substitution of one for the other constitutes a silent mutation.

A DNA sequence is "derived from" a source such as plastids or mitochondria if the DNA sequence exists in the source. The invention also includes variants of such a DNA sequence.

A DNA sequence is "functional" in plastids or mitochondria if it performs its expected function and is maintained in the progeny of the plastids or mitochondria.

A "plant cell protoplast capable of being transformed" means a protoplast that, after direct gene transfer, contains a plastid or mitochondrial genome comprising covalently linked DNA not found normally in that plastid or mitochondrion.

The plant cell protoplast capable of being transformed by the present method may be derived from cultured cells, cells that are attached to plant parts, or cells that are attached to multicellular plants. The transformable plant cell protoplasts may also be derived from unicellular plants such as algae. Some examples of unicellular plants include cyanobacteria (e.g. *Synechoccus* spp.), *Chlamydomonas*, and *Euglena*. The preferred plant cell protoplasts for the purpose of the present invention are derived from multicellular plants. After transformation, the protoplasts may be regenerated into plant cells.

Any plant cell protoplast may have its plastids or mitochondria genetically engineered by the present invention. Some examples of suitable plant cell protoplasts include *Solanum spp.* (potato), *Gossypium spp.* (cotton), *Glycine spp.* (soybean), *Petunia spp.* (petunia), *Daucus spp.* (carrot), *Citrus spp.* (orange, lemon), *Lycopersicon spp.* (tomato), *Brassica spp.* (turnip, cabbage, cauliflower, etc.), *Beta spp.* (beet), *Phaseolus spp.* (bean), *Helianthus spp.* (sunflower), *Arachis spp.* (peanut), *Amaranthus spp.* (grain Amaranth), *Medicago spp.* (alfalfa), *Trifolium spp.* (clover), *Atropa spp.* (nightshade), *Hyoscyamus spp.* (henbane), *Digitalis spp.* (foxglove), *Catharanthus spp.* (periwinkle), *Pisum spp.* (pea), and *Pinus spp.* (pine).

Plastids capable of being transformed include, for example, chromoplasts, amyloplasts, leucoplasts, etioplasts, proplastids, and chloroplasts. Chloroplasts are th preferred plastid for transformation.

The present invention also includes g nes that ar functional in plastids or mitochondria. The genes of the present invention confer desirable and useful traits on th plastids r mitochondria into which they are introduced. Some examples of useful traits include phytotoxin resistance, such as h rbicid or antibiotic resistance, improved phot synthetic efficiency, and enzym activity wher a chr mogenic substrate is known for the enzyme.

Herbicide resistance may be to any herbicide that is active in plastids or mitochondria. For example, many herbicides are active in chloroplasts. Such herbicides include the triazine, ur a, sulfonyl-urea, and uracil

classes of herbicides, as well as glyphosate (N-phosphonomethylglycine).

Some examples of triazine herbicides include atrazine, ametryn, metribuzin, and simazine. S me examples of urea herbicides include ph nylurea herbicides such as diuron, chloroxuron and flu rmeturon, and DCMU (dichl romethylurea). Some examples of sulfonylureas includ Oust and Glean. Som xamples of uracil herbicides include br macil and terbacil. These and ther herbicides ar described in L Baron and Gressel (1982).

It is not necessary to effect direct gene transfer to all of the approximately 50 to 100 chloroplasts in a cell with a gene encoding herbicide resistance in order to confer herbicide resistance on the cell. Direct gene transfer to a minor fraction of the chloroplasts is sufficient to protect the cells.

The ability to confer herbicide resistance, such as atrazine resistance, on plants is desirable for several reasons. It permits, for example, the use of a herbicide at increased levels on plants tolerant to the herbicide. Increased levels of the herbicide increase its efficacy in killing weeds.

Moreover, herbicide resistance can be used as a selectable marker genetically linked to a physiological trait that is more difficult to select. In order to take advantage of this possibility, a donor DNA plasmid is constructed that contains a gene conferring herbicide resistance and a second gene conferring another desirable trait. The second gene may confer, for example, improved photosynthetic efficiency. This donor DNA is introduced into a plant cell protoplast, which is regenerated into a plant cell culture and, eventually, a whole plant. The whole plant has both the herbicide resistance trait and the second trait. By growing the plant in the presence of the herbicide, one can select those plants having the second trait.

In addition, the introduction of a donor DNA that confers both herbicide resistance and a second agronomically useful trait on the chloroplasts of plants permits the second trait to be stably maintained in the chloroplasts by growing the plants in the presence of the herbicide. The instability of foreign genes in chloroplasts has already been noted by De Block et al. (1985) (see above).

Preferably, herbicide resistance is to atrazine which is 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine.

Antibiotic resistance is a trait that can be exploited for identifying *in vitro* the cells containing transformed plastids or mitochondria. Genes conferring this selectable marker are especially useful if they are genetically linked with agronomically useful traits. For example, resistance to chloramphenicol, kanamycin, or, in principle, any other antibiotic is useful in this way.

A trait useful primarily as a screenable marker in tissue culture for identification of plant cells containing genetically engineered plastids or mitochondria results from introduction of a gene encoding an enzyme having a chromogenic substrate. For example, if the enzyme is beta-galactosidase, the plant cells are plated on a tissue culture medium containing the chromogenic substrate Xgal (5-chloro-4-bromo-3-indolyl-beta-D-galactoside), and plant cells containing genetically engineered plastids or mitochondria are stained blue by the dye indigo released by betagalactosidase cleavage of Xgal.

The genes useful in the present invention may be obtained by methods known in the prior art. Such methods include isolating a natural gene or a variant of a gene that occurs in nature externally to the plastids or mitochondria into which the natural gene is to be introduced. The gene may be any natural gene or variant of a natural gene that is functional in plastids or mitochondria. Preferably, the gene is a natural plastid, mitochondrial or bacterial gene, and most preferably a chloroplast gene. In order to assure that the gene is indeed in the plastid or mitochondria and not in the nucleus, it is desirable, but not essential, that the gene be non-functional in the nucleus, or is at least significantly less functional in the nucleus than in the plastid or mitochondrion.

Some examples of natural bacterial genes include those encoding antibiotic resistance or enzymes having a chromogenic substrate. A bacterial gene capable of conferring antibiotic resistance on plastids or mitochondria is the chloramphenicol acetyl transferase gene. A bacterial gene encoding an enzyme having a chromogenic substrate is lacZ encoding betagalactosidase. Some examples of plastid or mitochondrial genes include those coding for herbicide resistance or for improved photosynthetic efficiency. A chloroplast gene capable of conferring herbicide resistance is the mutant psbA gene described by Hirschberg et al. (1983), or a mutant form of the psbD gene (Rochaix et al, 1984). A chloroplast gene capable of conferring improved photosynthetic efficiency is a modified form of rbcL, which codes for a modified large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). A gene that expresses the large subunit of Rubisco is already present in chloroplasts, and is involved in photosynthesis. It is possible, however, to introduce a modified (mutant, engineered, or heterologous) form of this gene with improved photosynthetic efficiency (Jordan et al., 1981).

One can also obtain a gene that is functional in plastids or mitochondria by constructing a chimeric gene. A chimeric gene is a gene at least one portion, preferably an active portion, of which is not covalently bound to the other portions in nature. The chimeric gene may either specify an RNA transcript or encode a polypeptide.

Th pr moter of the chimeric gene of the pr s nt inventi n may be any pr m ter that is functional in plastids r mitochondria. The pr moter of the chimeric gene may b derived fr m a natural plastid or mitochondrial gene, or it may b from a gene heterologous to plastids or mitochondria. The preferred natural plastid or mitochondrial gen promoters include those derived from psbA, psbD and rbcL genes. Th prom ter may also be a variant of a natural promoter. The heter l gous prom ter f the chimeric gene f the present invention may be a natural promoter that does not occur in nature in plastids or mitochondria.

Since the functions of plastid and mitochondrial genes are often similar or identical to those of bacterial genes, bacterial promoters may be functional in plastids or mitochondria. Any bacterial promoter functional in plastids or mitochondria may be used as the promoter of the chimeric gene of the present invention. Some suitable bacterial promoters include those from neomycin phosphortransferase II and from T-DNA genes such as the Ti plasmid nopaline synthase gene.

A heterologous promoter may also be any partially or wholly synthetic promoter that is functional in plastids or mitochondria. Partially or wholly synthetic promoters resembling their natural counterparts in having a TATAAT-like sequence at about −10 nucleotides from the start of transcription are also covered by this invention.

The 5′ untranslated region of the chimeric genes of the present invention may be any 5′ untranslated region that is functional in plastids or mitochondria. For example, the 5′ untranslated region may be derived from plastid or mitochondrial genes, or may be from genes heterologous to plastids or mitochondria. The preferred homologous 5′ untranslated region is that derived from psbA, psbD, or rbcL genes. The preferred heterologous 5′ untranslated regions are those derived from bacteria. Synthetic 5′ untranslated regions resembling their natural counterparts in having an effective ribosome binding site are also covered by this invention.

In principle, any coding region capable of confering a desirable trait on a plant cell is suitable for use as the coding region of the present invention. The coding region derived from any of the natural genes discussed above as well as from other sources may be used in constructing the chimeric genes of this invention.

Accordingly, the coding regions of this invention may be derived from natural plastid or mitochondrial genes, may be from a heterologous source, may be completely or partially synthetic, may be an in-frame fusion of two or more such coding regions. Each coding region encodes a polypeptide that confers one or more novel properties on the cells of plants.

An example of a polypeptide expressed by a gene that occurs naturally in the chloroplasts of certain plants is the mutant form of the 32kd polypeptide described by Hirschberg et al. (1983). This polypeptide was found to confer atrazine resistance on certain weeds. The psbA gene that encodes this polypeptide may be transferred to useful plants such as crop plants by the present invention.

An example of a coding region derived from a source heterologous to plastids and mitochondria is the coding region from plant, animal or bacterial *gsh*A and *gsh*B genes as well as that from a glutathione reductase (*gor*) gene, which can confer useful traits when introduced into plastids or mitochondria. *gsh*A and *gsh*B encode enzymes that catalyze the synthesis of the tripeptide glutathione, which is responsible for conjugative detoxification of many herbicides (Meister et al., 1983; Rennenberg, 1982).

Another example of a coding region derived from a source heterologous to plastids and mitochondria is the coding region from a plant, animal, insect, or bacterial glutathione-S-transferase gene. In some plants (Shimabukuro, et al., 1971) known to be tolerant to the herbicide atrazine, the basis of this tolerance is the presence of glutathione-S-transferase(s). The glutathione-S-transferases detoxify the phytotoxin by catalyzing the formation of an atrazine-glutathione conjugate.

Another coding region useful in the chimeric gene of the present invention encodes a more efficient form of Rubisco. Such coding regions may be derived from a natural gene.

The coding region of the present invention may also be derived from two or more different coding regions. Polypeptides encoded by such coding regions are called fusion polypeptides.

An example of a fusion polypeptide is a more efficient form of Rubisco. Rubisco has two functions — i.e. as a carboxylase and as an oxygenase. The carboxylase function is productive in photosynthesis, while the oxygenase function is undesirable. A suitable fusion polypeptide, accordingly, possesses a portion that maximizes the carboxylase function and a second portion that minimizes the oxygenase function.

The 3′ untranslated region of the chimeric gene either may occur naturally in a plastid or mitochondrial gene, or may be heterologous to the plastid or mitochondrial genome. The preferrd 3′ untranslated region is derived from a gene of plastid or mitochondrial origin. The preferred 3′ untranslated region is that of psbA, psbD or rbcL.

The transcribed regions of the genes of the present invention may specify RNA transcripts that are, in some cases, useful per se. These transcripts may constitute tRNA or rRNA. The transcript may also be an RNA that has a sequence complementary to at least a portion of the sequence of another RNA transcript. Such complementary sequences, known as anti-sense sequences, if long enough, may disrupt the function of the RNA sequence to which the anti-sense sequence is complementary, or may block transcription of that RNA from its gene; see Pestka et al., 1984; Melton, 1985; Izant et al., 1984; Simons et al., 1983; Coleman, 1984.

Transcribed regions useful in the present ivention may be derived from natural transcribed regions, or they may be wholly or partially synthetic. Anti-sense sequences may also be derived from at least a portion of a natural gene by reversing the orientation of that portion of the natural gene with respect to its promoter.

The genes suitable for use in the present invention are constructed in or inserted into a plasmid cloning vector by methods known in the art (Maniatis et al., 1982).

To integrate the foreign gene into the genomic DNA of the plant cell, it is advantageous if the gene is flanked by neutral DNA sequences (carrier DNA). The carrier DNA may consist of two linear DNA strands, so that the construct to be inserted into the plant cell is a linear DNA molecule. The construct for gene transformation can, however, also have a circular structure (plasmid structure). The carrier DNA can be of

synthetic origin or can be obtained from naturally occurring DNA sequences by treatment with suitable restriction endonuclease. Thus, for xample, naturally occurring plasmids which have been opened with one or more restriction endonucleases are suitable for use as carrier DNA. Exemplary of such a plasmid is the readily obtainable pUC8 plasmid (described by M ssing et al., 1982). Fragm nts of naturally occurring plasmids can also be used as carrier DNA.

The construct for transformation may or may not contain DNA from a Ti plasmid or from a modified Ti plasmid. A plasmid is considered to be a modified Ti plasmid if it contains at least one T-DNA border region. A T-DNA border region is a sequence of DNA in the genome of an Agrobacterium that causes a different sequence in the genome (the T-DNA) to be introduced into plant cells with which the Agro-bacterium is in contact. The T-DNA border region may, for example, be a sequence of a vector such as a Ti or Ri plasmid, or of a modified Ti or Ri plasmid. The T-DNA may be a sequence on the same vector as the border region or on a different vector.

The probability of the genetic transformation of a plant cell can be enhanced by different factors. Accordingly, as is known from experiments with yeast, the number of successful stable gene transformations increases

1. with the increasing number of copies of the new genes per cell,
2. when a replication signal is combined with the new gene. An integration signal is a signal that promotes the integration of a DNA strand into another DNA strand.
3. when an integration signal is combined with the new gene. An integration signal is a signal that promotes the integration of a DNA strand into another DNA strand.

The process of this invention is therefore susceptible of especially advantageous application when the transferred gene is combined with a replication signal which is effective in plant cells or with an integration signal which is effective in plant cells, or which is combined with both signals.

Protoplasts, cell culture cells, cells in plant tissues, pollen, pollen tubes, egg-cells, embryo-sacs or zygotes and embryos in different stages of development are representative examples of plant cells that are suitable starting materials for a transformation. Protoplasts are preferred on account of the possibility of using them directly without further pretreatments.

Isolated plant protoplasts, cells or tissues can be obtained by methods that are known per se or by methods analogous to known methods.

Isolated plant cells and tissues suitable as starting materials for obtaining isolated plant protoplasts can be obtained from any part of the plant, for example from leaves, embryos, stems, blossoms, roots or pollen. It is preferred to use leaf protoplasts. The isolated protoplast can also be obtained from cell cultures. Methods of isolating protoplasts are described e.g. in Gamborg et al. (1975).

The transfer of a new gene into plant cells is effected directly without infecting the cells with pathogens such as a plant pathogenic bacterium, virus or fungus, and without transfer of DNA by insects or fungi that are capable of infecting plants with DNA-transferring pathogens. This direct transfer is achieved by exposing the DNA comprising the gene to plant protoplasts in a medium in which the gene penetrates the protoplast and the plastids or mitochondria in the protoplast for a time sufficient to permit such penetration. The transformation frequency can be increased by combining this step with various techniques for gene transfer. Examples of such techniques include treatment with poly-L-ornithine, or poly-L-lysine, liposome fusion, DNA-protein complexing, altering the charge at the protoplast membrane, fusion with microbial protoplasts, or calcium phosphate coprecipitation and, in particular, by treatment with certain polyhydric alcohols such as polyethylene glycol, by heat shock and by electroporation, as well as by a combination of these last three mentioned techniques.

The medium suitable for use in the present invention may be any medium in which the DNA comprising the gene penetrates the protoplast and the plastids of mitochondria in the protoplast.

Suitable solutions into which the foreign gene and the receptor protoplasts are introduced are preferably the osmotically stabilized culture media employed for protoplast cultures.

Numerous culture media are already available which differ in their individual components or groups of components. However, the compositions of all media are in accordance with the principle that they contain a group of inorganic ions in the concentration range from about 10 mg/l to several hundred mg/l (so-called macroelements such as nitrate, phosphate, sulfate, potassium, magnesium, iron); a further group of inorganic ions in maximum concentrations of several mg/l (so-called microelements such as cobalt, zinc, copper, manganese); a number of vitamins (for example inositol, folic acid, thiamine); a source of energy and carbon, for example sucrose or glucose; and growth regulators in the form of natural or synthetic phytohormones of the auxin and cytokinin classes in a concentration range from 0.01 to 10 mg/l. The culture media are additionally stabilized osmotically with sugar alcohols (for example mannitol) or sugar (for example glucose) or salt ions (for example $CaCl_2$), and are adjusted to a pH in the range from 5.6 to 6.5.

A more detail d description of conventional culture media will be found, for example, in Koblitz et al. (1974).

A particularly suitable medium for the direct transformation of protoplasts comprises a polyhydric alc hol that is able to modify the protoplast membrane and is useful in promoting cell fusion. The preferred polyhydric alcohol is polyethylene glycol. Polyhydric alcohols of longer chain length, for example polypropyl ne glycol (425 to 4000 g/mole), polyvinyl alcohol or p lyhydric alcohols whose hydroxyl groups are partially or completely alkylated, may also be employed. The polyhydric detergents which are commonly

employed in agriculture and tolerated by plants, are also suitable polyhydric alcohols: such detergents are described in the following publications:

"McCutcheon's Detergents and Emulsifiers Annual" MC Publishing Corp., Ridgewood, New Jersey, 1981; Stache, H., "Tensid-Taschenbuch", Carl Hanser Verlag, Munich/Vienna, 1981.

The preferred polyhydric alcohol is polyethylene glycol having a molecular weight in the range from 1000 to 10,000 g/mole, preferably from 3000 to 8000 g/mole.

The frequency of direct transformation can be greatly improved by the techniques described in more detail below.

In the polyethylene glycol treatment, a suspension of the protoplasts may be added to a culture medium. The DNA comprising the gene, which may be linear or in the form of a circular plasmid, is then added to a mixture of polyethylene glycol and culture medium. Alternatively, protoplasts and DNA comprising the gene are first added to the culture medium and then polyethylene glycol is added.

In the process of this invention, electroporation and heat shock treatment have also proved to be particularly advantageous techniques.

In electroporation, Neumann et al. (1982) report that protoplasts are transferred to an osmoticum, for example a mannitol/magnesium solution, and the protoplast suspension is introduced into the electroporator chamber between two electrodes. By discharging a condenser over the suspension, the protoplasts are subjected to an electrical impulse of high voltage and brief duration, thereby effecting polarisation of the protoplast membrane and opening of the pores in the membrane.

In the heat treatment, protoplasts are suspended in an osmoticum, for example a solution of mannitol/calcium chloride, and the suspension is heated in small containers, for example centrifuge tubes, preferably in a water bath. The duration of heating will depend on the temperature chosen. In general, the temperatures are in the range of 40°C to 80° for 1 second to 1 hour. Optimum results are obtained at a temperature of 40° to 50° for 4 to 6 minutes, especially 45°C for 5 minutes. The suspension is subsequently cooled to room temperature or below.

It has also been found that the transformation frequency can be increased by inactivating the extracellular nucleases. Inactivation can be effected by using divalent cations that are tolerated by plants, for example magnesium or calcium. Preferably, the inactivation is effective by carrying out the transformation at a high pH value, with the optimum pH range being from 9 to 10.5.

Surprisingly, the selective use of these different methods results in a substantial increase in transformation frequency, which has long been an objective in the field of genetic engineering.

The lower the transformation frequency in gene transformation, the more difficult and time-consuming it is to find the few cloned cells resulting from the transformed cells from among the large number of non-transformed clones. Where the transformation frequency is low, the use of conventional screening techniques is virtually impossible, unless the gene employed is one with selective marker function (e.g. resistance to a specific substance). Low transformation frequency thus requires a very substantial investment in time and effort when using genes without marker functions.

By combating foreign gene and receptor protoplasts before employing the other techniques such as polyethylene glycol treatment, electroporation and heat shock treatment, one can bring about a significant improvement in transformation frequency as compared with a procedure in which the sequence of the steps employed is different.

A combination of two or three of the following techniques has proved advantageous: polyethylene glycol treatment, heat shock treatment and electroporation, with particularly good results being obtained by employing these techniques after the foreign gene and protoplasts have been introduced into a solution. The preferred technique is heat shock treatment before the polyethylene glycol treatment and the optional subsequent electroporation. In general, the additional electroporation effects a further increase in transformation frequency; but in some cases the results obtained by heat shock and polyethylene glycol treatment are no longer essentially improved by additional electroporation.

It is also possible to combine the use of divalent cations that are tolerated by plants and/or transformation at pH 9 to 10.5 with the individual transformation frequency movement techniques described above, namely polyethylene glycol treatment, heat shock treatment and electroporation.

Accordingly, the process of this invention permits a high transformation frequency to be achieved without utilizing pathogens such as viruses and Agrobacterium, or natural or modified Ti plasmids for the transformation.

An advantageous method comprises, for example, transferring protoplasts to a mannitol solution and mixing the protoplast suspension so obtained with an aqueous solution of the DNA comprising the gene. The protoplasts are then incubated in this mixture for 5 minutes at 45°C and subsequently cooled to 0°C over 10 seconds. After incubation, polyethylen glycol (mol. wt. 3000 to 8000) is added to th mixture until the concentration is in the rang from 1 to 25 %, preferably about 8 %. After cautious and thorough mixing, treatment is carried out in an electrop rator. Th protoplast suspension is then diluted with, and allowed to incubate in, culture medium in order to allow regeneration of its cell wall.

The process of this invention is suitable for the transformation of all plant cells, especially th se of the systematic gr ups Angiosp rmae and Gymnospermae.

Among th Gymnospermae, the plants of the Coniferae class are of particular inter st.

Among th Angiospermae, plants of particular interest are, in addition to deciduous trees and shrubs,

plants of the following families: Solanaceae, Cruciferae, Compositae, Liliaceae, Vitaceae, Chenopodiaceae, Rutaceae, Bromeliacea , Rubiaceae, Theaceae, Musaceae or Gramineae and of the order Leguminosae, in particular of the family Papilionaceae. Preferred plants ar r presentatives of the S lanacea , Cruciferae and Gramineae families.

Of particular interest ar plants of the genera Nicotiana, P tunia, Hyoscyamus, Brassica and Lolium, as f r example, *Nicotiana tabacum, Nicotiana plumbaginifolia, Petunia hybrida, Hyoscyamus muticus, Brassica napus, Brassica rapa* and *Lolium multiflorum*.

Plastids and mitochondria from all plants that can be produced by regeneration from protoplasts can also be usefully transformed by the process of this invention. Until now, it has not been possible genetically to manipulate plastids and mitochondria of representatives of the Gramineae family (grasses), which also comprises cereals such as, for example, maize, wheat, rice, barley, oats, millet, rye and sorghum. The present invention allows plastids and mitochondria of graminaceous cells, including cereal cells, to be transformed genetically by direct gene transformation. In the same way, it is possible to effect transformation of plastids and mitochondria of any cultivated plants such as those of the genera Solanus, Nicotiana, Brassica, Beta, Pisum, Phaseolus, Glycine, Helianthus, Allium, Triticum, Hordeum, Avena, Setaria, Oryza, Cydonia, Pyrus, Malus, Rubus, Fragaria, Prunus, Arachis, Secale, Panicum, Saccharum, Coffea, Camellia, Musa, Ananas, Vitis, Sorghum, Helianthus or Citrus.

Incorporation of transformed genes into plastids or mitochondria can be demonstrated by methods known in the prior art, for exmple by genetic crosses and molecular biological assays, including in particular the Southern blot analysis of plastid and mitochondrial DNA and by enzyme activity tests.

The Southern blot analysis can be carried out by example as follows: the DNA isolated from the plastids or mitochondria of transformed cells or protoplasts is electrophoresed in 1 % agarose gel after treatment with restriction enzymes, transferred to a nitrocellulose membrane [Southern et al. (1975)], and hybridized with in vitro labeled DNA whose existence it is desired to establish. DNA can be labeled in vitro to specify activity 5×10^8 to 10×10^8 c.p.m/micrograms by nick translation [Rigby et al., (1977)]. The filters are washed 3 times for 1 hour with an aqueous solution of 0.03 M sodium citrate and 0.3 M sodium chloride at 65°C. The hybridized DNA is visualized by autoradiography on X-ray film for one to several days.

The cells transformed with the desired gene are isolated by methods known in the prior art. These methods include selection and screening. Selection of nuclear genes is described by Fraley et al., (1983); Herrera-Estrella et al., (1983); and Bevan et al., (1983). Screening may be for beta-galactosidase [Helmer et al., (1984)], nopaline synthase, or octopine synthase [Wostemeyer et al, (1984); DeGreve et al., (1982)] or Atrazine resistance.

Prior to the present invention, it was not known that plastids or mitochondria could be transformed by direct gene transfer. Accordingly, it was not previously possible functionally to introduce desirable genes as isolated DNA into these organelles. Genes are considered to be functionally incorporated into the genome of plastids and mitochondria if they are capable of replication and expression.

An advantage of introducing genes into plastids or mitochondria by direct gene transfer is the possibility of simultaneously inactivating an undesirable gene that is already in the plastid or mitochondrial genome. This is possible since the process of direct gene transfer does not necessarily insert foreign genes into the plastid or mitochondrial genome by homologous recombination. For example, a donor DNA molecule carrying the atrazine resistant form of psbA will not necessarily insert into the correspondong atrazine sensitive psbA gene. On the other hand, because the plastid and mitochondrial genomes are relatively small, it is feasible to screen among transformed plant cells and find those in which the donor DNA insertion has randomly "hit" (inserted into) any desired function in the recipient genome. Direct gene transfer to plastid and mitochondrial genomes thus affords a way of inactivating a resident gene that was heretofore not feasible. By screening, for example, it is feasible to find transformed plant cells in which the atrazine sensitive psbA gene is inactivated by insertion of a donor DNA carrying an atrazine resistant psbA gene.

A further advantage of direct gene transfer into the genomes of plastids and mitochondria is that such genomes, unlike nuclear genomes, are maternally inherited through the cytoplasm, and are not usually transferred through pollen to their sexual progeny. Therefore, the possibility of transferring genes conferring desirable traits from crop plants to weeds is minimized.

Plant cells in culture transformed by the method of the present invention may be used to produce the polypeptide encoded by the inserted gene. Such products include, for example, the 32kd polypeptide expressed by psbA, chloramphenicol acetyl transferase, glutathione S-transferase, and the large subunit of Rubisco.

The plant cells containing the inserted gene may, in some cases, also be regenerated into multicellular plants that express the gene, and, therefore, have the desired new trait. Any whole plant capable of being regenerated from plant cells derived from protoplasts may be made by the method of the present invention. Some suitable whole plants includ , for example, *Solanum spp.* (potato), *Petunia spp.* (p tunia), *Daucus spp.* (carrot), *Lycopersicon spp.* (tomato), *Brassica spp.* (turnip, cabbage, cauliflower, etc.), *Medicago spp.* (alfalfa), *Trifolium spp.* (cl ver), Citrus, atropa, Hyoscyamus, Salpiglossis, Arabidopsis, Digitalis, Cichorium, Gossipium, Glycine, Geranium, Antirrhinum, and Asparagus. The plants may be regenerated by methods known in the art; see Evans and Bravo, 1983; Dale, 1983. For xample, plants may be regenerated from any suitable propagule, such as cells, calli, tissue, organs, buds, cuttings, shootlets, rootlets, plantlets, somatic embryos, and the like.

The present invention further includes seeds of plants produced by the method of this invention as long as the seeds contain the inserted gene and the desirable trait resulting therefrom. Progeny of plants produced by the method of this invention, including sexual and vegetative progeny, are further embodiments. Sexual progeny may result from selfing or crosspolination. Such seeds and progeny of herbicid resistant or tolerant parents containing unstable herbicide resistance or tolerance will retain their herbicide resistance or tolerance as long as they are grown in the presence of the herbicide. The preferred herbicide is atrazine. Accordingly, the present invention permits the production of genetically engineered plants capable of surviving treatment with a herbicide, such as atrazine, at concentrations sufficient to be lethal to sensitive plants.

It goes without saying that the present invention also includes all plant protoplasts, plant cells, plant tissue cultures propagating material or plants transformed by the method described herein, as well as any plant derived from a material which was transformed by the method described herein, which derived plant is still showing the trait or traits resulting from said transformation.

Examples

The procedures of the Examples given below may generally be found in Maniatis et al., 1983. Enzymes can be obtained from New England Biolabs, and are used according to the manufacturers recommendations unless otherwise indicated.

Example 1: Construction of pCAT° (See Figure 1).

1. Vector plasmid pMON 187 carrying the Km^r gene from Tn 903 is digested with Hind III and Bam HI.
2. A promoterless CAT gene is isolated as a gel-purified Hind III/Bam HI fragment from pSVO plasmid (Gorman et al., 1982).
3. Ligation of the fragment from step 2 to the fragment from step 1 affords a plasmid that is transformed into HB101 E. Coli selecting for Ap^r. A Km^s colony has the plasmid with the structure given in Figure 1 for pCAT°.

Example 2: Alternative Construction of pCAT°.

Example 2 may be repeated with plasmid other than pMON 187.

A suitable plasmid containing the Km^r gene from Tn 903 may be constructed as follows.

A 1.2 kb Ava II fragment, containing the Km^r gene from Tn 903 is isolated from the plasmid pA02 [Oka, et al., (1981)]. The ends of the Ava II fragment are filled in with Klenow polymerase, and Bam HI linkers are ligated to the blunt ended fragment. This DNA is then restricted with the enzymes Taq I and Bam HI and is ligated into the plasmid pBR327, previously digested with ClaI and Bam HI. Recombinants containing the Tn903 fragment confer Km^r on E. coli.

Example 3: Construction of p32 CAT (see Figure 1)

The following steps are performed in order to add the psbA promoter to the CAT° promoterless gene:

4. pCAT° is digested with Sma I and Hind III;
5. A gel-purified 161 bp Sma I/Hind III fragment containing the psbA promoter is isolated from plasmid pAH484. The recombinant plasmid pAH484 contains the 3.68 kb EcoRI fragment of chloroplast DNA from herbicide (atrazine)-resistant *Amaranthus hybridus* cloned into pBR322 [Hirschberg and McIntosch, (1983)].
6. The fragment isolated in step 5 is added to the larger fragment resulting from step 4. Ligation leads to p32CAT, which confers Cm^r on transformed E. Coli.

Example 4: Construction of Plasmid pUCHI; a Vector Carrying psbA and a Chimeric psba/CAT Gene Construction. (See Figure 2)

The plasmid pUCHI was constructed in the pUC8 vector in five steps (7–11):

7. (Figure 1) A 3.6 kb EcoRI fragment (gel purified) containing the complete psbA gene is isolated from pAH484, described above.
8. pUC8 (Figure 2) is linearized at its unique EcoRI site.
9. The linearized pUC8 fragment from step 8 is ligated to the fragment described in step 7. Transformation of E. coli affords Ap^r colonies that possess the desired insert fragment, and one was designated pUC8-32.
10. A gel-purified 1.8 kb Xho I/Bam HI fragment was isolated from p32CAT.
11. The plasmid resulting from step 9, pUC8-32, was digested partially with Sal I and to completion with Bam HI. Ligation with the fragment resulting from step 10 and selection for Cm^r after transformation of E. coli lead to a colony of E. coli containing pUCHI.

Example 5: Construction of pBRCAT Containing a Chimeric psbA/CAT Gene. (See Figure 3)

The construction of pBRCAT is accomplished by ligating together the following three DNA fragments:

a. The Eco RI/Hind III (promotor) fragment (ca. 660 bp) from the psbA gene in plasmid pAH484 (step 12);
b. The Hind III/Bam HI fragment of pSVO containing the promoterless CAT gene (step 2);
c. EcoRI/Bam HI digested pBR322 as vector (step 13).

14. Ligation leads to a plasmid, pBRCAT which confers Cm^r on E. coli host cells into which it is introduced

by transformation.

Example 6: Introduction of Donor DNA into Plant Protoplasts

Tobacco protoplasts at a population density of 2×10^6 per ml are suspended in 1 ml of K_3 medium [q.v. Z. Planzenphysiologi *78*, 435–455 (1976); Mutation Research *81* 165–175 (1981)], containing 0.1 mg/l of 2.4-dichlorophenoxyacetic acid, 1.0 mg/l of 1-naphthylacetic acid and 0.2 mg/l of 6-benzylaminopurine. Protoplasts are obtained from an enzyme suspension by flotation on 0.6 molar sucrose at pH 5.8 and subsequent sedimentation (100 g for 5 minutes) in 0.17 M calcium chloride at pH 5.8. to this suspension are added, in succession, 0.5 ml of 40 % polyethylene glycol (PEG) with a molecular weight of 6000 in modified (adjusted again to pH 5.8 after autoclaving) F-medium [Nature *296*, 72–74 (1982)] and 65 microliters of an aqueous solution containing 15 micrograms of the donor DNA (p32CAT, pUCHI, or pBRCAT) and 50 micrograms of calf thymus DNA. This mixture is cultured for 30 minutes at 26°C with occasional agitation and subsequent stepwise dilution with F medium. The protoplasts are isolated by centrifuging (5 minutes at 100 g) and resuspended in 30 ml of fresh K_3 medium. Further incubation is carried out in 10 ml portions in Petri dishes of 10 cm diameter at 24°C and in the dark. The population density is 6.3×10^4 protoplasts per ml. After 3 days the culture medium in each dish is diluted with 0.3 parts by volume of fresh K_3 medium and incubated for a further 4 days at 24°C and 3000 lux. After a total of 7 days, the clones developed from the protoplasts are embedded in a culture medium solidified with 1 % agarose and containing 10 mg/l chloramphenicol and cultured at 24°C in the dark by the bead type culture method [Plant Cell Reports, *2*, 244–247 (1983)]. The culture medium is replaced every 5 days by fresh nutrient solution of the same kind.

After 3 to 4 weeks of continued culturing in chloramphenicol containing culture medium, the resistant calli of 2 to 3 mm diameter are transferred to agar-solidified LS culture medium (Physiol. Plant *18*, 100–127 (1965)], containing 0.05 mg/l of 2,4 dichlorophenoxyacetic acid, 2 mg/l of 1-naphthylacetic acid, 0.1 mg/l of 6-benzylaminopurine, 0.1 mg/l of kinetin and 10 mg/l chloramphenicol. Chloramphenicol resistant *Nicotiana tabacum* Petit Havana SRI plants are obtained by inducing shoots on LS medium containing 10 mg/l chloramphenicol and 0.2 mg/l of 6-benzylaminopurine, and subsequent rooting on T medium [Science *163*, 85–87 (1969)].

Selection for chloramphenicol resistant calli and plants is performed essentially as described in De Block et al., (1984)

Example 7: Screening for Atrazine-resistant Chloroplast Transformants.

a. Callus

An atrazine toxicity curve is determined for untransformed callus at a concentration range of 1–10 μM atrazine with various light intensities. Callus isolated from putative chloroplast transformants and control callus are placed on nutrient agar plates with an atrazine concentration and light intensity that completely bleaches chlorophyl from control tissue. The resistance to atrazine is visually assayed by the continued greening of resistant tissue.

b. Whole Plants

Plants derived from chloroplast transformants are assayed for atrazine resistance by the following methods:

Chlorophyl fluorescence induction in leaves. When electron transport on the reducing side of photosystem II is inhibited, as by atrazine, chlorophenyl-absorbed radiant energy is reemitted as fluorescence. This fluorescence can be detected at the leaf surface. Fluorescence is detected from leaf disks clamped horizontally over a transparent lucite window, as described by Malkin et al. (1981). Exciting light is supplied from a dc-operated projector, filtered, resulting in a band of actinic light from 500 to 600 nm with an intensity about 10 $nE\times cm^{-2}\times s^{-1}$. The exciting light time is 3 ms. The exciting light is directed vertically to the leaf surface and fluorescence from the same surface is collected by a flexible light guide, passed through a cut-off filter (660 nm) to a red-sensitive photo-multiplier. The fluorescence transients are recorded on an oscilloscope and directly filmed.

c. Light modulated flotation

When disks excised from leaves are floated on a phosphate buffer containing surfactant, they continue to float as long as photosynthesis occurs by maintaining a high ratio of O_2 to CO_2 in the intercellular air spaces. If the leaf disks are floated in the dark, or if photosynthesis inhibiting herbicides are added to the medium, they rapidly lose buoyancy and sink. A method of assaying for atrazine resistance is described by Hensley (1981). Leaf disks are transferred to tubes containing a solution of atrazine and placed under vacuum. The leaf disks are rapidly infiltrated by the solution and sink to the bottom of the solution. The vacuum is then released, a bicarbonate solution is added, and the tubes placed under light. When photosynthesis is not inhibited by atrazine, photosynthetically generated oxygen within the tissue restore buoyancy and the disks float to the surface. Atrazine-sensitive disks remain at the bott m.

Example 8: Transformation of Cells of Brassica rapa c.v. Just Right

Brassica rapa protoplasts are washed with a suitable osmoticum and suspended in a p pulation density of 5×10^6 per ml in a culture medium prepared according to Protoplasts 83, Proceedings Experientia Supplementum, Birkhäuser Verlag, Basel, Vol. 45 (1983), 44–45. 40 % p lyethylene glycol (PEG) with a molecular weight of 6000, dissolv d in modified F medium (pH 5.8) (q.v. Exampl 1b), is mixed with the

protoplast suspension to a final concentration of 13 % PEG. To this mixture is added immediately a soluti n of 50 micrograms of plasmid pBRCAT or p32CAT digested with endonuclease SalI, 60 micr grams of water. With occasional agitation, the mixture is incubated for 30 minutes at 20°–25°C. Then 3×2 ml f m dified F m dium (6 ml in all) and 2×2 ml of cultur medium (4 ml in all) are added at 5 minute intervals. The protoplast suspension is transferred to 10 cm Petri dishes and made up to a total volume of 20 ml with additional culture medium. These protoplast suspensions are incubated in the dark for 45 minutes at 26°C. The protoplasts are isolated by sedimentation for 5 minutes at 100 g, taken up in an initially liquid and then solidifying agarose gel culture medium and culture by the bead type culture method [Plant Cell Reports, 2, 244–247 (1983)]. After 4 days, in the development stage of the first cell division, chloramphenicol is added to the cultures in a concentration of 10 mg/l. The liquid culture medium surrounding the agarose segments is replaced every 4 days by fresh chloramphenicol containing nutrient solution. After 4 weeks the chloramphenicol-resistant clones are isolated and then further cultured by providing them weekly with chloramphenicol-containing nutrient solution (10 mg/l).

Example 9: Transformation of Protoplasts of Graminaceous Plants of the Species Lolium multiflorum

Protoplasts of Lolium multiflorum (Italian regrass) are taken up at a concentration of 2×10^6 per ml of 0.4 molar mannitol at pH 5.8. To this suspension are added, in succession, 0.5 ml of 40 % polyethylene glycol (PEG) with a molecular weight of 6000 in modified (pH 5.8) F medium [Nature *296*, 72–74 (1982)], and 65 microliters of an aqueous solution containing 50 micrograms of the plasmid p32CAT or pBRCAT. This mixture is incubated for 30 minutes at 26°C with occasional agitation and subsequently diluted with F medium, as described [Nature *296* (1982), 72–74]. The protoplasts are isolated by centrifugation (5 minutes at 100 g) and taken up in 4 ml of CC culture medium [Potrykus, Harms, and Lörz, Callus Formation from Cell Culture Protoplasts of Corn (Zea Mays L.), Theor. Appl. Genet. *54*, 209–214 (1979)] and incubated in the dark at 24°C. After 14 days the developing cell cultures are transferred to the same culture medium, but with chloramphenicol (10 mg/l). Resistant cell colonies are transferred to agar medium (the same medium as above, 10 mg/l) chloramphenicol without osmoticum) and, after reaching a size of several grams fresh weight per colony, analyzed for the presence of the bacterial gene and for the biological activity of the gene.

Example 10: Transformation of Culture Cells of Nicotiana tabacum by Transferring p32CAT, pUCHI or pBRCAT by means of electroporation.

Protoplasts are produced by sedimentation from 50 ml of a log phase suspension culture of the nitrate reductase deficiency variant of *Nicotiana tabacum*, cell strain nia-115 [Müller, A.J. and R. Grafe, Mol. Gen. Genet. *161*, 67–76 (1978)], and resuspended in 20 ml of enzyme solution [2 % Cellulase Onozuka R-10, 1 % Macerozyme R019 and 0.5 % Driselase (available from Chemische Fabrik Schweizerhalle, Basel) in a wash solution 0.3 M mannitol, 0.04 M calcium chloride and 0.5 % 2-(N-morpholino) ethanesulfonic acid), adjusted to pH 5.6 with KOH] and incubated for 3 hours on a gyratory shaker at 24°C. The protoplasts are then separated from undigested tissue by filtering them through a 100 micrometer mesh sieve. An equal volume of 0.6 M sucrose is added and the suspension is centrifuged for 10 minutes at 100 g. The protoplasts floating on the surface are collected and washed 3 times by sedimentation in the wash solution.

Transformation is carried out by electroporation. The chamber of a Dialog® "Porator" (available from Dialog GmbH, Harffstr. 34, 4000 Düsseldorf, West Germany) is sterilized by washing with 70 % ethanol and then 100 % ethanol and dried by a current of sterile air from a laminar flow hood). The protoplasts are suspended at a concentration of 1×10^6/ml in 0.4 M mannitol solution, adjusted with magnesium chloride to a resistance of 1.4 kOhm and pBRCAT, pUCHI, or p32CAT DNA is added in a concentration of 10 microgram/ml. 0.38 ml samples of this protoplast suspension are subjected 3 times at 10 second intervals to a charge of 1000 volts or to a charge of 2000 volts. The protoplasts are then cultured in a concentration of 1×10^6/ml in 3 ml of AA-CH medium [AA medium of Glimelius, K. et al., Physiol. Plant. *44*, 273–277 (1978)], modified by increasing the inositol concentration to 100 mg/l and the sucrose concentration to 34 g/l, as well as by adding 0.05 ml/l of 2-(3-methyl-2-butenyl)adenine, and which is solidified by a 0.6 % content of agarose (Sea Plaque, FMC Corp., Marine Colloids Division, P.O. Box 308, Rockland, Maine 0481, USA). After 1 week, the agarose layer containing the protoplasts is transferred to 30 ml of liquid AA-CH medium which contains 10 mg/l chloramphenicol. After 3 weeks, during which time half the liquid medium is replaced weekly by fresh medium of the same composition, the transformed cell colonies can be observed visually. Four weeks after being transferred to the medium containing chloramphenicol, these cell colonies are transferred to AA medium [Glimelius, K. et al., Physiol. Plant. *44*, 273–277 (1978)]; with 0.8 % agar which contains 10 mg/l of chloramphenicol, for further culturing and investigation.

Analogous assays with protoplast of *Brassica rapa* and *Lolium multiflorum* also result in successful transformations.

Example 11: Transformation of Chloroplasts of Cells of Nic tiana tabacum *by Transfer of the Donor DNA by Electroporation.*

The preparation of the electroporator is as described in Example 10 and of the protoplasts as in Example 6.

For transformation, protoplasts of *Nicotiana tabacum* are r suspended in a concentration of 1.6×10^6/ml in mannitol solution (0.4 M, buffered with 0.5 % w/v of 2(N-morpholino)ethanesulfonic acid; pH 5.6). The resistance of the protoplast suspension is measured in the p rator chamber (0.38 ml) and adjusted to 1 to 1.2

kOhm with magnesium chloride solution (0.3 M). 0.5 ml samples are put into capped plastic tubes (5 ml volume) to each of which are added initially 40 microliters of water containing 8 micrograms of donor DNA and 20 micrograms of calf thymus DNA, and 0.25 ml of polyethylene glycol solution (25 % w/v in 0.4 M mannitol). Nine minutes after addition of the DNA, 0.38 ml portions are put into the pulse chamber and 10 minutes after the addition of DNA, the protoplast suspensions present in the chamber are subjected to 3 impulses (1000–2000 volts) at 10 second intervals. The treated portions are put into Petri dishes of 6 cm diameter and kept for 10 minutes at 20°C. Then 3 ml of K_3 medium containing 0.7 % w/v of Sea Plaque agarose are added to each Petri dish and the contents of the dish are thoroughly mixed. After solidification of the contents of each dish, the cultures are kept for 1 day at 25°C in the dark and then for 6 days in light. The protoplast-containing agarose is then cut into quarters and introduced into liquid medium. The protoplasts are then cultured by the bead type culturing method. Callus tissues, which are obtained by selection of the transformed material with chloramphenicol, and plants regenerated therefore contain the CAT enzyme (chloramphenicol acetyltransferase) as product of the CAT gene.

Electroporation induces a 5- to 10-fold increase in the frequency of transformation compared with the method without electroporation. Analogous assays with *Brassica rapa* c.v. Just Right and *Lolium multiflorum* also bring about an increase in the frequency of transformation of the same order of magnitude.

Example 12: Transformation of Nicotiana tabacum *by Transfer of the CAT Gene by Means of Heat Shock*

Protoplasts isolated from leaves or cell cultures of *Nicotiana tabacum* are isolated as described in Examples 6 and 10 and transferred to an osmotic medium as described in the preceding Examples. The protoplast suspensions are kept for 5 minutes at 45°C, cooled with ice for 10 seconds and then the plasmid pBRCAT, pUCHI, or p32CAT is added as described in Examples 6 and 10. The heat shock treatment increases the transformation frequency by a factor of 10 or higher compared with a transformation carried out without this treatment.

Example 13: Transformation of Different Plant Cells by Transfer of the CAT Gene by Combining Protoplasts and Gene as First Step and Subsequent Combined Treatment.

Protoplsts of the plants:
Nicotiana tabacum c.v. Petit Havana SRI (*A*)
Brassica rapa c.v. Just Right (*B*) and *Lolium multiflorum* (*C*)
are isolated and transferred to an osmotic medium as described in Example 11. The protoplast suspensions are mixed with the plasmid pUCHI, p32CAT or pBRCAT as described in Examples 6 to 9, but without simultaneous treatment with polyethylene glycol. The protoplast suspensions are then subjected to a heat shock treatment as described in Example 12, then to a polyethylene glycol treatment as described in Examples 6 to 9, and finally subjected to electroporation as described in Example 11. The transformation frequency in this procedure is in the range from 10^{-3} to 10^{-2}, but may be from 1 to 2 % depending on the conditions.

References

Barton, et al., Cell, *32*, 1033 (1983).
Barton and Chilton, Methods in Enzymology, *101*, 527 (1984).
Bevan, et al., Nature, *304*, 184 (1983).
Bevan, et al., Nucleic Acid Res., *12*, 8711 (1984).
Boliva, et al., Gene *2*, 75 (1977).
Chilton, et al., Genetics, *83*, 609 (1976).
Chilton, "Plant Gene Vectors", in "The Role of Plant Biotechnology in Plant Breeding", Report of the 1984 Plant Breeding Research Forum, August 21–23 (1984) Page 177–192 (1985).
Coleman, et al., Cell, *37*, 429 (1984).
Comai, et al., Plasmid, *10*, 21 (1983).
Dale, "Protoplast Culture and Plant Regeneration of Cereals and Other Recalcitrant Crops" in Protoplasts 1983 Lecture Proceedings, Potrykus et al., (eds), Experientia Supplement, Volume 46, Birkhäuser, Basel, (1983).
De Block, et al., EMBO J. *4*, 1367 (1985).
De Block, et al., EMBO J. *3*, 1681 (1984).
De Greve, et al., Nature, *300*, 752 (1982).
De Framond, et al., Biotechnology, *1*, 266 (1983).
Ditta, et al., Proc. Natl. Acad. Sci USA *77*, 7347 (1980).
Evans and Bravo, "Protoplast isolation and Culture" in Handbook of Plant Cell Culture, Volume 1, Evans et al. (Eds), McMillian Publishing Co., New York, (1983).
Fraley, et al, Proc. Natl. Acad. Sci., *80*, 4803 (1983).
Fraley, et al., Biotechnology, *3*, 629 (1985).
Fraley, et al., Plant Molecular Biology, *3*, 371 (1984).
Gamborg, Plant Physiol., *45*, 372 (1970).
Gamborg, et al., Plant Tissue Culture Methods, 11 (1975).
Garfinkel, et al., Cell, *27*, 143 (1981).

G rman, et al., Molecular and Cellular Biology, *2*, 1044 (1982).
Helmer, et al., Biotechnology, *2*, 520 (1984).
Hensley, Weed Sci., *29*, 70 (1981).
Hepburn, et al., J. Mol. Appl. Genet., *2*, 211 (1983).
Hernalsteens, et al., Nature, *287*, 654 (1980).
Herrera-Estrella, et al., Nature, *303*, 209 (1983).
Hirschberg and McIntosch, Science, *222*, 1346 (1983).
Hirschberg, et al., Z. Naturforsch., *396*, 412 (1984).
Hoekema, et al., Nature, *303*, 179 (1983).
Holsters, et al., Mol. Genet., *163*, 181 (1978).
Horsch, et al., Science, *227*, 1229 (1985).
Izart, et al., Cell, *36*, 1007 (1984).
Jordan and Ogren, Nature, *291*, 513 (1981).
Jorgensen, et al., Mol. Gen. Genet, *177*, 65 (1979).
Klee, et al., Biotechnology, *3*, 637 (1985).
Koblitz, Kulturpflanze, XXII, 93 (1974).
Le Baron and Gressel, Herbicide Resistance in Plants, John Wiley and Sons (1982).
Maliga, Z., Pflanzenphysiol., *78*, 453 (1976).
Malkin, et al., Plant Physiol., *67*, 570 (1981).
Maniatis, et al., Molecular Cloning, Cold Springs Harbor Laboratory (1982).
Matzke and Chilton, J. Mol Appl. Genet., *1*, 39 (1981).
Messing, et al., Gene, *19*, 269 (1982).
Meister et al., Ann Rev. Biochem., *52*, 711 (1983).
Melton, Proc. Natl. Acad. Sci., *82*, 144 (1985).
Murashige, et al., Physiol Plant, *15*, 473 (1962).
Neumann, et al., EMBO J., *7*, 841 (1982).
Norrander, et al., Gene *26*, 101 (1983).
Oka, et al., Nature, *276*, 845 (1978).
Oka, et al., J. Mol. Biol., *147*, 217 (1981).
Paszkowski, et al, EMBO J., *3*, 2717 (1984).
Pestka, et al., Proc. Natl. Acad. Sci., *81*, 7525 (1984).
Rennenberg, Phytochemistry *21*, 2771 (1982).
Rigby et al., J. Mol. Biol., *113*, 237 (1977).
Roberts, Plant Molecular Biology Reporter, *3*, 107 (1985).
Rochaix, et al., Plant Mol. Biol., *3*, 363 (1984).
Schilperoort et al., European Patent Application *86*, 537 (1983).
Shimabukuro, et al., Plant Physiol., *47*, 10 (1971).
Simons, et al., Cell, *34*, 683 (1983).
Southern, et al., J. Mol. Biol., *98*, 503 (1975).
Wang, et al., Cell, *38*, 455 (1984).
Wostemeyer, et al., Mol. Gen., *194*, 520 (1984).
Yadav, et al., Proc. Natl. Acad. Sci. USA, *79*, 6322 (1982).
Zambryski, et al, EMBO J., *2*, 2143 (1983).

CLAIMS

1. A process for introducing DNA comprising one or more genes having a promoter that is functional in plastids or mitochondria directly into plastids or mitochondria in a plant cell protoplast, the process comprising exposing the DNA to the protoplast in a medium in which the DNA penetrates the protoplast and the plastids or mitochondria in the protoplast for a time sufficient to permit such penetration, without exposing the protoplast to a pathogen.

2. The process of claim 1 further comprising the step of regenerating plant cells from the transformed protoplasts.

3. The process of claim 2 further comprising regenerating plants from the plant cells.

4. The process of claim 1 wherein the DNA is linear.

5. The process of claim 1 wherein the DNA is a plasmid.

6. The process of claim 5 wherein the plasmid does or does not contain one or more T-DNA border regi ns.

7. The process of claim 1 wherein th DNA comprises a gene that confers herbicide resistance on the plastids and mitoch ndria.

8. The process of claim 7 wherein the herbicide is atrazine.

9. The process of claim 7 wh rein th DNA confers herbicide resistance and a sec nd agronomically useful trait.

10. The process of claim 1 wherein th DNA comprises a selectable mark r gene and a gen that confers an agronomically useful trait.

11. The process of claim 10 wherein the selectabl marker gene confers antibiotic resistance.
12. The process of claim 11 wherein the antibiotic is chloramphenicol r kanamycin.
13. The process of claim 10 wherein the agronomically useful trait is herbicide resistance.
14. The process of claim 13 wherein the herbicid is atrazine.
15. The process of claim 1 wherein the gene is chim ric.
16. The process of claim 1 wherein the DNA comprises a replication signal.
17. The process of claim 1 wherein the DNA comprises an integration signal.
18. The process of claim 1 wherein the plant cell protoplast is a leaf cell protoplast.
19. The process of claim 1 wherein the medium is an osmotically stabilized protoplast culture medium.
20. The process of claim 1 wherein the medium comprises divalent cations tolerated by plants.
21. The process of claim 20 wherein the divalent cations are magnesium or calcium cations.
22. The process of claim 1 wherein the medium comprises a polyhydric alcohol that is able to modify the protoplast membrane and to promote cell fusion.
23. The process of claim 22 wherein the polyhydric alcohol is polyethylene glycol, polypropylene glycol or polyvinyl alcohol.
24. The process of claim 23 wherein the polyhydric alcohol is polyethylene glycol.
25. The process of claim 24 wherein the polyethylene glycol has a molecular weight of 1000 to 10 000.
26. The process of claim 1 wherein the DNA and protoplasts are subjected to heat shock.
27. The process of claim 1 wherein the DNA and protoplasts are subjected to electroporation.
28. The process of claim 1 wherein the DNA is introduced into the protoplast by a combination of at least two of polyethylene glycol treatment, heat shock, and electroporation.
29. A process according to claim 28, wherein the gene transfer is carried out by introducing the foreign gene and the protoplasts into a solution and subsequently subjecting the resultant suspension first to heat shock treatment and then to treatment with polyethylene glycol.
30. A process according to claim 28 wherein the gene transfer is carried out by introducing the foreign gene and the protoplasts into a solution and subjecting the resultant suspension first to heat shock treatment, then to treatment with polyethylene glycol, and finally, to electroporation.
31. The process of claim 1 wherein the medium comprises a polyhydric alcohol that is able to modify the protoplast membrane and to promote cell fusion and the DNA and protoplasts are subjected to at least one of electroporation or heat shock.
32. The process of claim 1 further comprising the step of inactivating extracellular nucleases.
33. The process of claim 1 wherein the protoplasts are from plant cells of the Gramineae, Solanaceae, or Cruciferae families.
34. The process of claim 33 wherein the protoplasts are from plant cells of the Gramineae family.
35. The process of claim 34 wherein the plant cells of the Gramineae family are cells that produce cereals.
36. The process of claim 35 wherein the cereal is maize, wheat, rice, barley, oats, millet, rye or sorghum.
37. Plant protoplasts, plant cells, plant tissue cultures, propagating plant material or plants transformed by the process of claim 1.
38. Any plant derived from a transformed material of claim 37 still showing the trait or the traits resulting from said transformation.

Printed for Her Majesty's Stationery Office by Croydon Printing Company (UK) Ltd, 4/87, D8991685.
Published by The Patent Office, 25 Southampton Buildings, London WC2A 1AY, from which copies may be obtained.